Published in final edited form as:

J Immunol Methods. 2007 December 1; 328(1-2): 204–214.

Hock immunization: A humane alternative to mouse footpad injections

T. Kamala

Building 4, Room 111, LCMI, NIAID, NIH, 9000 Rockville Pike, Bethesda, MD 20892,
tkamala@niaid.nih.gov Ph.: 301-435-5684, Fax: 301-496-4286

Abstract

Footpad injection is a commonly used immunization method in mice. Being relatively easy to do with well-characterized lymphatic drainage, it has become a very useful immunization protocol to study local immune responses in draining lymph nodes. However, its disadvantages include use of only hind feet as a routine site of immunization since mice use their fore feet for food handling, and exacerbation of inflammation and swelling at the injection site leading to unrelieved pain and distress since feet are weight-bearing structures. With increasingly stringent Institutional guidelines for animal manipulations, there is increasing need for more humane protocols. A novel immunization protocol involving injection into the hock, the lateral tarsal region just above the ankle, a non-weight bearing structure draining to the same lymph node as the footpad, retains the advantages of footpad immunization without its drawbacks. This study, comparing immune responses between footpad and hock immunization in six different inbred mouse strains to two different protein antigens and a heat-killed bacterium, shows that hock immunization is a better alternative to footpad immunization, inducing comparable immune responses and being considerably more humane.

Keywords

Footpad immunization; alternative; Hock immunization; CFA; IACUC regulations; immune response

1. Introduction

The mouse footpad is a commonly used injection site for studying many in vivo and in vitro immunological phenomena. There are several reasons for this.

First, following injection of antigen into the footpad, the subsequent swelling, called the Delayed Type Hypersensitivity Reaction, can be used as a convenient, relatively objective, sensitive, quick, and simple assay to measure in vivo immune responses (Gray and Jennings, 1955).

Second, footpad injection is also a mainstay for more sensitive and quantitative *in vitro* measurements of immune responses. This is because the path of lymph drainage from footpad injections is well known. Footpad injection is a combination of intradermal and subcutaneous injections, with the lymph draining directly up the hind leg to the popliteal lymph node

Correspondence: T. Kamala, Building 4, Room 111, 9000 Rockville Pike, Bethesda, MD 20892. E-mail: tkamala@niaid.nih.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

(Kawashim.Y et al., 1964) and then mainly to the medial iliac (Van den Broeck et al., 2006) (sometimes referred to as peri-aortic) nodes, and also somewhat to the subiliac (or inguinal) nodes (Tilney, 1971). Harvesting these three nodes draining the site of footpad injection provides a relatively large number of responding lymphocytes that can then be studied in various ways. Thus for immunologists studying local cellular immune responses to nominal protein antigens, injection into mouse footpads with the given protein emulsified in an adjuvant such as Complete Freund's Adjuvant (CFA) provides enlarged, easily accessible draining lymph nodes, with sufficient numbers of lymphocytes to enable detailed analyses.

Third, in the immunotoxicity field, the well-defined lymph drainage from footpad injections has enabled the development of the popliteal lymph node assay (PLNA) (Gleichmann, 1981). In its simplest form, this assay measures the enlargement of the popliteal lymph node six to eight days after footpad injection, to distinguish between immunostimulating and inert chemicals (Ravel and Descotes, 2005).

Fourth, for generating high titer antibodies to an antigen, immunizing mice in the footpad with an antigen-CFA emulsion, followed by boosts at the base of the tail is a very commonly used protocol for production of antibodies.

Since mice use their forefeet for handling food, Institutional Animal Care and Use Committees (IACUCs) generally prohibit the use of the fore feet for footpad injections. However, because the hind feet are major weight bearing structures, the inflammation and swelling that typically occurs at the site of injection leads to progressive debilitation. A mouse thus injected in one of its hind feet becomes progressively unable to bear weight on its injected foot, sometimes resulting in lameness. Thus, although immunizing mice footpads with antigen in CFA or other adjuvants may provide a robust immune response, such immunizations are questionable from the humane point of view, as they cause visible pain to the injected mice, and sometimes even lameness. Given such outcomes, most Institutional Animal Care and Use Committee guidelines limit CFA injection to a single footpad per mouse. Some Institutions even prohibit the use of mouse footpad immunizations altogether.

This paper describes a novel method wherein mice are immunized in the hock, and compares the resulting immune response with the immune response elicited by footpad immunization. Immunizing mice in the hock, the lateral tarsal region just above the ankle, is a protocol that retains the advantages of footpad immunization in that the immune response is directed to the same draining lymph nodes without the incidental impairment of mobility. Such an immunization has already been applied to study local immune responses in sheep (Heath and Brandon, 1983; Kerlin and Watson, 1987a; Kerlin and Watson, 1987b). This paper shows that hock immunization compares favorably with footpad immunization in terms of strength and quality of the induced immune response to protein antigens while being much more humane, inducing significantly less impairment of mobility.

2. Material and Methods

2.1 Animals

Age-matched 8 to 12 week old mice were used in all experiments. A/JCr, BALB/c/AnNCr, CBA/JCr, and C57Bl/6NCr mice were obtained from NCI-DCT, Frederick, MD. B10.BR/Ai and B10.D2/nSnAi mice were obtained from Taconic Farms, Germantown, New York. Animals were housed in specific-pathogen free conditions and were used in accordance with NIH Animal Care and Use guidelines. The NIH is an AAALAC accredited facility.

2.2 Immunization protocol

Adjuvant preparation—Complete Freund's Adjuvant (CFA) from SIGMA (Cat. # F-5881) contains the heat-killed *Mycobacterium tuberculosis* strain, H37Ra, in mineral oil at a concentration of 1 mg/ml. The CFA was mixed thoroughly by vortexing to ensure that the heat-killed bacteria were incorporated in the suspension. Immediately after vortexing, the adjuvant was drawn into a glass syringe using a 19-gauge needle. Bubbles were carefully eliminated from the syringe and the needle removed. **Antigen preparation:** The protein antigens, ovalbumin (OVA; cat. # A-5503) and human alpha lactalbumin (hALAC; cat. # L-7269) were purchased from Sigma. (St. Louis, MO.) as lyophilized powders. The proteins were reconstituted in 1X PBS (phosphate buffered saline) to a concentration of 1 mg/ml. **Making the emulsion:** The protein-CFA emulsions were prepared by mixing together the protein solution with the CFA suspension at a ratio of 1: 1, using two glass syringes, one loaded with the adjuvant, and the other with the antigen solution in PBS, connecting them with a 3 way stop cock. The approximately 200–250µl dead volume in the 3-way stopcock was eliminated by carefully and slowly pushing the protein solution through the stopcock right until the other edge, at which point the syringe containing the CFA was attached. Care was taken to first slowly introduce the protein solution into the CFA suspension drop by drop before mixing thoroughly. The protein-CFA emulsion was tested for readiness by putting a drop of emulsion onto PBS. When the emulsion is done, it will stay intact as a tight drop on the PBS. If the emulsion is not done, then it will start to spread out on the PBS with globs of oil radiating out from the center.

Each mouse was injected subcutaneously in either the left hind footpad or in the left hind hock with 20µl of the CFA emulsion containing 10 µg of protein (Fig. 1). For studying systemic antigen-specific antibody levels, mice were boosted with protein emulsion in IFA (Incomplete Freund's Adjuvant, Sigma Chemical Co., St. Louis, MO., Cat. # F-5506) prepared similarly to the procedure used for preparing protein-CFA mixtures. The booster dose of 10 µg protein was administered subcutaneously near the base of the tail three weeks after priming.

Hock-immunization protocol—For hock immunization in the left hind foot, the mouse is placed on the rack underneath the cage top and held gently by just the left foot with the top down. To do the hock injection with a CFA emulsion, a right-handed person would hold the left hind foot between the left thumb and left middle finger leaving the left index finger free. Holding the loaded syringe and needle ready in the right hand, inject laterally on the right side (inside part of the leg) just above the ankle into the space indicated in Fig. 1.

For footpad immunization in the left hind foot, the mouse is placed on the rack underneath the cage top and held gently by just the left foot with the top down and the ventral part of the foot facing up.

For the injections, a 27-gauge needle was used and the injection site approached with the bevelled edge of the needle facing to the left in the case of hock immunization, and downwards in the case of footpad immunization, i.e. the bevel facing the skin while going in with the needle. The needle was pushed into the skin a little way past the bevel before starting to inject. After finishing to inject, the needle was slowly withdrawn from the injection site while pressing down lightly on top of the injection site with the left index finger. Once the needle was completely withdrawn from the injection site, it was pressed down a little harder for a few seconds, maybe 2 to 3 seconds, and then released. At this point, there should be no injected material oozing from the site of the injection. If material was still oozing out, the injection site was pressed down for a few seconds more.

2.3 Assays for CD4+ T cell purification and functions

Preparation of cell suspensions—Eight to ten days after priming, the mice were euthanized by cervical dislocation. The draining lymph nodes (popliteal, subiliac [inguinal] and medial iliac [or peri-aortic]) were harvested. Lymph node single cell suspensions were made by rubbing and pressing the lymph nodes between two layers of sterile nylon mesh using a pair of sterile forceps.

Negative selection to obtain highly pure populations of CD4 + T cells—First the cells were counted and an aliquot taken for pre-depletion flow cytometry staining. Then the cells were incubated for half an hour at room temperature with a cocktail of biotinylated antibodies specific for B cells (anti-B220, clone RA3-6B2; anti-CD19, clone 6D5; anti-IgM, clone RMM-1), CD8 T cells (anti-CD8a, clone 53-6.7; anti-CD8b, clone 53-5.8), dendritic cells (anti-CD11c, clone N418), NK/NK T cells (anti-CD49b, clone DX5), macrophages (anti-FcγRII and FcγRIII, clone 93), MHC class II (clone M5/114) and gamma delta T cells (anti-γδ TCR, clone GL3). All the antibodies except anti-CD8b and anti-γδ were from BioLegend, CA. Anti-CD8b and anti-γδ antibodies were from BD Biosciences Pharmingen, CA. After washing, the cells were incubated with Cellaction Biotin Binder beads (DynaL Biotech, Invitrogen, CA) for half an hour at room temperature. Bead bound cells were removed by placing the cell suspension in a magnet and collecting the unbound supernatant. From each cell sample, an aliquot was taken for post-depletion flow cytometry staining. Cell surface Fc receptor staining was blocked by incubation with cocktail containing equal mixtures of rat, mouse and hamster sera plus 10 μg/ml of purified rat anti-mouse Fc receptor (FcR; clone 2.4G2). Flow cytometry staining using anti-CD3e FITC (clone 17A2, BioLegend, CA), anti-CD4 (clone RM4-5 PE, BD Biosciences Pharmingen, CA) and 7AAD (7-amino Actinomycin, BD Biosciences Pharmingen, CA), indicated that CD4 + T cell purity of ≥97% was routinely achieved using this homemade cocktail. Dead cells were excluded from analysis by gating on Forward Scatter positive, 7-AAD negative events. Flow cytometry was also used to determine the percentage of CD4+ T cells in each pre-depletion lymph node sample, and using the total pre-depletion lymph node count, the pre-depletion CD4+ T cell count for each sample was derived.

Antigen-specific CD4+ T cell proliferation assay—50,000 lymph node CD4+ T cells were cultured with 100,000 irradiated (3000 rads) T –depleted spleen cells with indicated graded doses of antigens in a water jacketed 5%CO₂ incubator for four days in round bottom 96 well plates. The culture medium was advanced DMEM-F12 (Invitrogen Corp., San Diego, CA) supplemented with 2mM glutamine, 5% fetal calf serum, 50μg/ml gentamicin, 100U/ml penicillin, 100μg/ml streptomycin and 12.5μM beta-mercaptoethanol. The cultures were pulsed with tritiated thymidine for the final 12 hours of culture. Stimulator cells were obtained from control mice of the same strain for each experiment and were depleted of CD4+ and CD8 + T cells using biotinylated anti-Thy 1.2 (CD90.2; clone 30 H12, BioLegend, CA) antibody and Cellaction Biotin Binder beads (DynaL Biotech, Invitrogen, CA).

Antigen-specific CD4+ T cell cytokine secretion—Supernatants from duplicate cultures to those set up for proliferation were harvested after 72 hours and stored at - 80°C. ELISAs (Enzyme Linked Mono Sorbent Assays) for mouse IFN-γ and IL-17A were performed using RnD ELISA kits (RnD Systems, Minneapolis, MN).

To control for the efficacy of immunization in each experiment, cell cultures were set up in parallel to measure the proliferation and cytokine secretion responses to PPD (Purified Protein Derivative, Mycos Research, LLC, Loveland, CO), which is derived from the H37Rv strain of *Mycobacterium tuberculosis*, a micro-organism closely related to the micro-organism present in the adjuvant used in all the immunizations.

2.4 Antigen-specific systemic antibody responses

Mice were bled from the tail vein into heparinized (20U/ml; American Pharmaceutical Partners, Los Angeles, CA) PBS at least two weeks after being boosted. The blood cells were spun down and the plasma stored at -80°C . Immulon II HB plates (Thermo Electron Corporation, Franklin, MA. Cat # 3455) were coated with either H139.52.1 mAb clone (rat anti-mouse kappa, Southern Biotech, cat# 1180-01), or OVA or hALAC at a final conc. of $10\mu\text{g/ml}$ in 1X PBS with $100\mu\text{l/well}$. Plates were left in the refrigerator overnight. After washing with 1X PBS with 0.05% Tween 20 and 0.0025% BSA, the plates were blocked with $200\mu\text{l}$ per well of 1% BSA for 1 to 1 and 1/2 hours at RT and were washed again. The following reagents were used to generate standard curves for each antibody isotype being measured: For IgG1, clone MOPC-31C (BD Biosciences Pharmingen, cat# 557273); for IgG2a, clone G155-178 (BD Biosciences Pharmingen, cat# 553454); for IgG2c, clone SF1-1.1 (BD Biosciences Pharmingen, cat# 553563); for IgG2b, clone MPC-11 (BD Biosciences Pharmingen, cat# 557351); for IgG3, clone A112-3 (BD Biosciences Pharmingen, cat# 553486).

Each standard concentration was set up in triplicate at $100\mu\text{l/well}$. At least three dilutions were tested per antibody isotype per sample except in the case of IgG3 where two dilutions per sample were tested. Wells coated with anti-kappa antibody, H139.52.1, received antibody standards. Wells coated with protein antigens received plasma dilutions. Samples and standards were incubated at room temperature for 1 to 1 and 1/2 hours. After washing the plates thoroughly, $100\mu\text{l/well}$ of detecting antibodies were added to each well. The following detecting antibodies were used: For IgG1: clone X56-HRP (BD Biosciences Pharmingen, cat# 559626); for IgG2a and IgG2c: clone R19-15-HRP (BD Biosciences Pharmingen, cat# 553391); for IgG2b: clone R12-3-Biotin (BD Biosciences Pharmingen, cat# 553393); for IgG3: clone R40-82-Biotin (BD Biosciences Pharmingen, cat# 553401). All detecting antibodies were used at 1: 1000 dilution in PBS-1% BSA and at $100\mu\text{l/well}$. After incubating at room temperature for 1 to 1 1/2 hours, plates were washed. For biotinylated detecting antibodies, 1:1000 dilution of streptavidin (SA)- Horse Radish Peroxidase (HRP) in PBS-1%BSA was added at $100\mu\text{l/well}$. Plates were incubated for 30 minutes at room temperature and then washed. $100\mu\text{l/well}$ of HRP substrate (ABTS from KPL Laboratories, Gaithersburg, MD, prepared according to manufacturer's instructions) was added to all plates. Plates were incubated at room temperature in the dark. Readings were performed using a Vmax Kinetic plate reader (Molecular Devices, Sunnyvale, CA) set at 405nm.

2.5 Assessment of mobility

Groups of BALB/c and B10.D2 mice were immunized in either the left hind footpad or left hind hock with OVA-CFA. In order to objectively record differences relating to mobility between footpad- and hock-immunized mice, the mice were daily monitored and scored by the animal care technicians in a blinded fashion. The following scores were used for mobility assessment [adapted from (Morton and Griffiths, 1985; Morton, 2000)]:

1. Normal Gait
2. Barely discernible lameness
3. Obvious limp through partial weight bearing
4. Unable to bear weight

Intermediate scores (1.5, 2.5) were used occasionally when mice displayed a behaviour interpreted by the technician to be between the above described definitions.

2.6 Statistical Analysis

Dose response curves, and mobility scores were compared using GraphPad's Prism 4.0 for Macintosh (GraphPad Software, Inc., San Diego, CA). Cell numbers, percentages and antibody titers between footpad- and hock-immunized mice were compared using GraphPad's t-test calculator for unpaired t-test (<http://www.graphpad.com/quickcalcs/ttest1.cfm>). Numbers within parentheses in the text are averages $\pm$ standard error of the mean.

3. Results

Six different inbred strains of mice (BALB/c, B10.D2, B10.BR, CBA, A and C57Bl/6) were immunized either in the footpad or hock with two different protein antigen-adjuvant emulsions (ovalbumin-complete Freund's adjuvant [OVA-CFA] and human alpha lactalbumin-CFA [hALAC-CFA]). The resulting local immune responses in the draining lymph node were analyzed and the following characteristics were compared between footpad- and hock-immunized mice: CD4⁺ T cell percentages, and absolute number of lymph node cells and numbers of CD4⁺ T cells; CD4⁺ T cell proliferation responses; CD4⁺ T cell cytokine secretion responses and systemic antigen-specific IgG1, IgG2a, IgG2b, IgG2c and IgG3 levels.

3.1 Comparing total lymph node cell numbers, and CD4⁺ T cell percentages between footpad- and hock- immunized mice

In order to carefully compare the CD4⁺ T cell responses in footpad- and hock - immunized mice, three measures of lymph node response, namely, the CD4⁺ T cell percentage, the total lymph node number, and the CD4⁺ T cell number were assessed.

Percentages of CD4 T cells: In 7/11 cases, the percentage of CD4 T cells were significantly higher in hock-immunized than in footpad-immunized mice, whereas there were no statistically significant differences in the remaining four cases.

Total lymphocyte numbers in the draining lymph nodes: For three of the six mouse strains (B10.D2, B10.BR and C57Bl/6), footpad and hock immunizations resulted in similar expansion/retention of cells in the draining lymph nodes, whereas in the remaining three strains (BALB/c, CBA, A) there was significantly less total expansion/retention following hock immunizations (Fig. 2 B, E).

Total CD4 T cell numbers: Overall the number of CD4⁺ T cells was significantly higher in footpad-injected mice in about half the cases.

3.2 Comparing CD4⁺ T cell proliferation responses between footpad- and hock-immunized mice

Overall, CD4⁺ T cells from footpad- versus hock-injected mice made similar proliferative responses (Fig. 3) and strain-specific differences in responsiveness were consistent and reproducible for both types of injections. Also BALB/c, B10.D2 and B10.BR mice made robust proliferation responses while responses of CBA, A and C57Bl/6 mice were weaker. These differences between strains could be due to different kinetics of response and such differences need to be studied further to support the hock approach.

3.3 Comparing CD4⁺ T cell cytokine secretion responses between footpad- and hock-immunized mice

The three strains that gave robust proliferation (BALB/c, B10.D2 and B10.BR) also produced considerable amounts of IFN- γ and IL-17. Cytokine secretion from CD4⁺ T cell cultures generated from CBA, A and C57Bl/6 mice were very low (average of 30 to 50 pg/ml) and did

not show clear dose responses. Therefore, they were not analyzed further. The same was true for IL-4, IL-6, IL-10 and TNF- α in BALB/c, B10.BR, B10.D2 and C57Bl/6 mice.

With three exceptions, the IFN- γ and IL-17 cytokine responses of CD4⁺ T cells from the three analyzable strains were not significantly different for footpad- and hock-immunized mice. BALB/c CD4⁺ T cells from footpad-immunized mice made significantly higher amounts of IFN- γ and IL-17 to PPD (2 μ g/ml) compared to CD4⁺ T cells from hock-immunized mice ($p = 0.04$ and $p = 0.01$, respectively for IFN- γ and IL-17) (Fig. 4 insets). The B10.D2 response to PPD showed similar trends. However, the differences were not significant, possibly because the overall IFN- γ levels were much lower than those obtained from BALB/c T cells.

3.4 Comparing systemic antibody responses between footpad- and hock-immunized mice

Mice from all six strains were immunized either in the footpad or hock with 10 μ g of either OVA-CFA or hALAC-CFA. Two to three weeks later, all the mice were boosted with the same dose of the priming antigen in IFA administered near the base of the tail. Mice were tail bled at least two weeks after the boost and the plasma stored at -80°C . Systemic antigen-specific IgG1, IgG2a, IgG2b, IgG2c and IgG3 titers were measured using a quantitative ELISA protocol.

The pattern of antigen-specific antibody responses among the tested strains conformed to published reports. For example, it has been previously shown that B10.BR mice make strong anti-hALAC IgG antibody responses, followed by B10.D2 mice and BALB/c mice, and that C57Bl/6 mice make the weakest responses (Horiuchi et al., 1990). We observed the same patterns (Fig. 5). Likewise, anti-OVA IgG antibody responses have been reported to be strongest in CBA/J mice, followed by BALB/c mice and C57Bl/6 mice (Takeyoshi and Inoue, 1992), and we saw a similar result for all IgG isotypes except IgG1 where one mouse out of five each of footpad- and hock-immunized C57Bl/6 mice had higher amounts of IgG1.

The antibody titers were generally similar between footpad- and hock-immunized mice, with a few exceptions. Hock-immunized B10.BR mice had significantly higher IgG2a and IgG2b titers to hALAC compared to footpad-immunized mice (Fig. 5), and hock-immunized BALB/c had significantly higher IgG1 titers to OVA than footpad-immunized mice. The reverse was true for OVA-CFA immunized A/J mice (Fig. 5).

3.5 Assessment of mobility in BALB/c and B10.D2 mice immunized in either the footpad or hock with OVA-CFA

Footpad immunization clearly induced greater impairment of mobility compared to hock immunization even though the mobility scores were strain-specific (Fig. 6) with greater effects in B10.D2 mice ($p < 0.0005$) compared to BALB/c mice ($p < 0.005$) as has been reported previously (Liang et al., 2006). Interestingly, in B10.D2 but not in BALB/c mice, starting at day 8 post-footpad immunization, impairment of mobility increased again. The mobility scores of hock-immunized BALB/c and B10.D2 mice were much lower all the way through. In fact, hock-immunized mice had no impairment in their mobility during the entire time course of observation except very slight impact on days 3, 4, 10 and 11 post-hock immunization in B10.D2 mice.

4. Discussion

Footpad immunization is one of the most commonly used methods of immunization in mice. Given increasing animal welfare concerns regarding unrelieved pain and distress associated with this immunization, there is a need to develop a more humane immunization protocol that is equally easy, targets the same draining lymph nodes, and induces comparable immune

responses. Hock immunization is a novel protocol that retains the benefits of footpad immunization, yields comparable immune responses and induces much less impairment of mobility.

Based on the results described in this study, for six inbred mouse strains, and three different antigens (two proteins and a heat-killed bacterium), hock-immunization induced local CD4⁺ T cell responses that were comparable to those induced by footpad immunization indicating that it is suitable for studying local immune responses in draining lymph nodes. While there were interesting and significant differences in the absolute numbers of lymphocytes and CD4⁺ T cells in the draining lymph nodes following footpad- or hock-immunizations, overall the pattern that emerged was the following. While absolute number of cells tended to be significantly higher in footpad-immunized mice, CD4⁺ T cell percentages tended to be significantly higher in hock-immunized mice (Fig. 2) suggesting that hock immunizations might lead to preferential accumulation, retention or proliferation of CD4⁺ T cells in draining lymph nodes compared to footpad immunizations. In two of the three strains where such a pattern emerged, namely, CBA and A mice, the responses to the immunizations were weak, with low proliferation counts and very low levels of cytokine secretion. While CBA (Hogaboam et al., 1998; Jilek et al., 2001) and A (Vaz et al., 1971) are known to be poor responders to OVA, their responses to hALAC have not been reported previously. On the other hand when a strong immune response was induced by the immunization (in BALB/c mice), and yet more cells were harvested from footpad- compared to hock-immunized mice, the ensuing responses were still comparable in terms of CD4⁺ T cell proliferation and cytokine secretion. Overall, CD4⁺ T cell proliferation, and IFN- γ and IL-17 cytokine responses were similar between footpad- and hock-immunized mice.

CD4⁺ T cell responses of C57Bl/6 mice were comparatively weaker, regardless of the route of immunization. Since C57Bl/6 is one of the most commonly used inbred strains of mice in immunological research, it is important to consider why this should be so. C57Bl/6 mice lack a functional MHC class II I-E (Jones et al., 1981; Mathis et al., 1983) which has been previously correlated with reduced immune responsiveness to certain proteins such as pigeon cytochrome c (PCC) (Schwartz et al., 1976; Solinger et al., 1979), Hen Egg Lysozyme (HEL) (Hill and Sercarz, 1975) and Hepatitis B antigen (HBsAg) (Neurath et al., 1983). Further, C57Bl/6 antigen presenting cells (APCs) were shown to be poor in inducing anti-staphylococcal enterotoxin B (SEB) responses from SEB-specific T cell clones (Robinson et al., 1991) suggesting that it is possible that fewer peptides from OVA and hALAC are presented in C57Bl/6 than in other strains thereby generating a lower magnitude of response. One reason for the recent increase in popularity of C57Bl/6 mice in immunological models in spite of its relatively poor CD4 response is the explosion in gene targeting studies employing the “knock out” technology. To accomplish this, 129 mouse substrains’ embryonic cells (ES) are most commonly used since they tend to most extensively colonize the host embryo (Simpson et al., 1997). Usually the resultant transgenic ES cells are micro-injected into blastocysts of C57Bl/6 because it is highly fecund, more extensively characterized than 129 and has a contrasting coat colour (Mogil and Wilson, 1997). Further, the anti-OVA T cell proliferation responses observed in C57Bl/6 mice in this study (Fig. 3) correlate well with those reported in the published literature (Lundberg et al., 1992; Kweon et al., 1999; DePaolo et al., 2003) even though these studies used 10 times more protein for immunization compared to the present study which used only 10 μ g, and they used a linear scale to represent proliferation data even though proliferation being an exponential function is best represented using a log scale as has been done here. Finally, C57Bl/6 response to PPD was comparable to the response in other strains indicating that the lower C57Bl/6 response was protein-specific and could be overcome by strongly immunogenic proteins such as those derived from mycobacteria.

Alternatives to footpad immunization for immunotoxicological evaluations have been described previously including injection in the dorsal part of the foot (Leenaars et al., 1995) or intradermally on the top of the head, in the midline between the eyes (Nierkens et al., 2004). Immunization in the dorsal part of the foot is not a suitable alternative to footpad immunization because they have similar resultant pathological effects implying that dorsal foot immunization may induce pain and distress, and impairment of mobility similar to footpad immunization. While intradermal injections in the ear or head area draining to the submandibular (or auricular) lymph nodes appear to be more humane alternatives for DTH measurements and for immunotoxicological evaluations of drugs and other chemicals, respectively, they cannot be considered an alternative for injection of protein-adjuvant mixtures which induce much more severe inflammation. Since antigen-specific CD4⁺ T cell responses in the draining lymph nodes following a single injection of protein-adjuvant mixture into the hock were remarkably similar to those induced by footpad immunization, single injections of chemicals or drugs in the hock could be considered as a promising alternative for immunotoxicological assays using lymph node assays.

Systemic antibody responses were generally comparable between footpad- and hock-immunized mice for the most part. In two cases, namely, in the response of B10.BR mice to hALAC, and in the response of BALB/c mice to OVA, the response in hock-immunized mice was significantly stronger compared to footpad-immunized mice while in one case, namely, in the response of A/J mice to OVA, the response of footpad-immunized mice was significantly stronger compared to hock-immunized mice. Overall, the pattern of antibody response to both OVA and hALAC in the different mouse strains also correlated well with those reported in the published literature (Horiuchi et al., 1990; Takeyoshi and Inoue, 1992). In these earlier studies too antibody responses of C57Bl/6 mice to OVA and hALAC were comparatively lower.

Thus hock immunization of protein-adjuvant mixtures induced systemic antibody responses comparable or greater than those induced by footpad immunization. Therefore, hock immunization should be considered as a more humane alternative for production of antibodies.

In summary, hock immunization was clearly much more humane in terms of its effects on mobility compared to footpad immunization while the immune responses induced by these immunizations were comparable both in terms of the local CD4⁺ T cells, and in terms of the systemic antibody levels, in six different inbred mouse strains, analyzing the response to two protein antigens, and a heat-killed bacterium. These results suggest that hock immunization shows promise as a better alternative to footpad immunization for studying local and systemic immune responses.

Acknowledgements

I thank Dr. Polly Matzinger for supporting this study, for suggesting hock as an alternative to footpad injections, and for suggesting I look at immune responses in A/J mice. I thank Dr. Ted Torrey for taking the picture of hock injection; Dr. David Usharauli for taking the picture of footpad injection, and for suggesting advanced DMEM-F12 medium as an alternative to IMDM medium for mouse CD4⁺ T cell culture; Mr. Abhi Bhirud for assisting me in the mouse tail vein bleeds; Mr. Ricardo Dreyfuss for helping to put together Fig. 1. I acknowledge and thank Drs. Polly Matzinger, Ron Schwartz and Ted Torrey for their critical comments on the manuscript. I thank Dr. Charles (Garry) Linton and Ms. Sonia Farmer for helping with the mobility assessment, and Ms. Priscella Nimako, Ms. Maria Jorge and Ms. Martha Delgado for monitoring footpad- and hock-injected mice for mobility. Finally, I thank the NIAID Animal Care and Use Committee (ACUC) for their encouragement of and support for this study. This work was supported by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

References

- DePaolo RW, Rollins BJ, Kuziel W, Karpus WJ. CC chemokine ligand 2 and its receptor regulate mucosal production of IL-12 and TGF-beta in high dose oral tolerance. *J Immunol* 2003;171:3560–3567. [PubMed: 14500652]

- Gleichmann H. Studies on the mechanism of drug sensitization: T-cell-dependent popliteal lymph node reaction to diphenylhydantoin. *Clin Immunol Immunopathol* 1981;18:203–211. [PubMed: 6451341]
- Gray DF, Jennings PA. Allergy in experimental mouse tuberculosis. *Am Rev Tuberc* 1955;72:171–195. [PubMed: 13238778]
- Heath T, Brandon R. Lymphatic and blood vessels of the popliteal node in sheep. *Anat Rec* 1983;207:461–472. [PubMed: 6650876]
- Hill SW, Sercarz EE. Fine specificity of the H-2 linked immune response gene for the gallinaceous lysozymes. *Eur J Immunol* 1975;5:317–324. [PubMed: 61870]
- Hogaboam CM, Lukacs NW, Chensue SW, Strieter RM, Kunkel SL. Monocyte chemoattractant protein-1 synthesis by murine lung fibroblasts modulates CD4+ T cell activation. *J Immunol* 1998;160:4606–4614. [PubMed: 9574568]
- Horiuchi T, Yagi J, Asano Y, Tada T. Epitope-specific regulation of the antibody response against alpha-lactalbumins in the mouse. *Int Arch Allergy Appl Immunol* 1990;92:408–415. [PubMed: 1707403]
- Jilek S, Barbey C, Spertini F, Cortes B. Antigen-independent suppression of the allergic immune response to bee venom phospholipase A(2) by DNA vaccination in CBA/J mice. *J Immunol* 2001;166:3612–3621. [PubMed: 11207323]
- Jones PP, Murphy DB, McDevitt HO. Variable synthesis and expression of E alpha and Ae (E beta) Ia polypeptide chains in mice of different H-2 haplotypes. *Immunogenetics* 1981;12:321–337. [PubMed: 6937437]
- Kawashim Y, Sugimura M, Hwang YC, Kudo N. Lymph System in Mice. *Japanese Journal of Veterinary Research* 1964;12:69-&.
- Kerlin RL, Watson DL. Effect of dextran sulphate on IgG subclass of antibody in efferent popliteal lymph of sheep. *Immunol Cell Biol* 1987a;65(Pt 5):411–417. [PubMed: 2449393]
- Kerlin RL, Watson DL. The secondary immune response to *Staphylococcus aureus* vaccines in efferent popliteal lymph of sheep. *Immunology* 1987b;60:295–301. [PubMed: 3817874]
- Kweon MN, Fujihashi K, Wakatsuki Y, Koga T, Yamamoto M, McGhee JR, Kiyono H. Mucosally induced systemic T cell unresponsiveness to ovalbumin requires CD40 ligand-CD40 interactions. *J Immunol* 1999;162:1904–1909. [PubMed: 9973457]
- Leenaars PP, Hendriksen CF, Koedam MA, Claassen I, Claassen E. Comparison of adjuvants for immune potentiating properties and side effects in mice. *Vet Immunol Immunopathol* 1995;48:123–138. [PubMed: 8533308]
- Liang DY, Guo T, Liao G, Kingery WS, Peltz G, Clark JD. Chronic pain and genetic background interact and influence opioid analgesia, tolerance, and physical dependence. *Pain* 2006;121:232–240. [PubMed: 16516386]
- Lundberg K, Dencker L, Gronvik KO. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) inhibits the activation of antigen-specific T-cells in mice. *Int J Immunopharmacol* 1992;14:699–705. [PubMed: 1521937]
- Mathis DJ, Benoist C, Williams VE 2nd, Kanter M, McDevitt HO. Several mechanisms can account for defective E alpha gene expression in different mouse haplotypes. *Proc Natl Acad Sci U S A* 1983;80:273–277. [PubMed: 6296871]
- Mogil JS, Wilson SG. Nociceptive and morphine antinociceptive sensitivity of 129 and C57BL/6 inbred mouse strains: implications for transgenic knock-out studies. *Eur J Pain* 1997;1:293–297. [PubMed: 15102394]
- Morton DB. A systematic approach for establishing humane endpoints. *Ilar J* 2000;41:80–86. [PubMed: 11406701]
- Morton DB, Griffiths PH. Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. *Vet Rec* 1985;116:431–436. [PubMed: 3923690]
- Neurath AR, Stark D, Strick N, Sproul P. H-2 linked genetic control of immune responsiveness to hepatitis B surface antigen (HBsAg) in mice. *J Med Virol* 1983;12:227–236. [PubMed: 6418854]
- Nierkens S, Nieuwenhuijsen L, Thomas M, Pieters R. Evaluation of the use of reporter antigens in an auricular lymph node assay to assess the immunosensitizing potential of drugs. *Toxicol Sci* 2004;79:90–97. [PubMed: 14976356]
- Ravel G, Descotes J. Popliteal lymph node assay: facts and perspectives. *J Appl Toxicol* 2005;25:451–458. [PubMed: 15986413]

- Robinson JH, Pyle G, Kehoe MA. Influence of major histocompatibility complex haplotype on the mitogenic response of T cells to staphylococcal enterotoxin B. *Infect Immun* 1991;59:3667–3672. [PubMed: 1910013]
- Schwartz RH, David CS, Sachs DH, Paul WE. T lymphocyte-enriched murine peritoneal exudate cells. III. Inhibition of antigen-induced T lymphocyte Proliferation with anti-Ia antisera. *J Immunol* 1976;117:531–540. [PubMed: 1084902]
- Simpson EM, Linder CC, Sargent EE, Davisson MT, Mobraaten LE, Sharp JJ. Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. *Nat Genet* 1997;16:19–27. [PubMed: 9140391]
- Solinger AM, Ultee ME, Margoliash E, Schwartz RH. T-lymphocyte response to cytochrome c. I. Demonstration of a T-cell heteroclitic proliferative response and identification of a topographic antigenic determinant on pigeon cytochrome c whose immune recognition requires two complementing major histocompatibility complex-linked immune response genes. *J Exp Med* 1979;150:830–848. [PubMed: 92520]
- Takeyoshi M, Inoue T. H-2 haplotype and sex-related differences in IgG response to ovalbumin in mice. *Jikken Dobutsu* 1992;41:315–319. [PubMed: 1505623]
- Tilney NL. Patterns of lymphatic drainage in the adult laboratory rat. *J Anat* 1971;109:369–383. [PubMed: 5153800]
- Van den Broeck W, Derore A, Simoens P. Anatomy and nomenclature of murine lymph nodes: Descriptive study and nomenclatory standardization in BALB/cAnNCrI mice. *J Immunol Methods* 2006;312:12–19. [PubMed: 16624319]
- Vaz NM, Phillips-Quagliata JM, Levine BB, Vaz EM. H-2-linked genetic control of immune responsiveness to ovalbumin and ovomucoid. *J Exp Med* 1971;134:1335–1348. [PubMed: 5112206]

Fig. 1.
Immunization schematic

Fig. 2.

Comparison of CD4+ T cell percentage (A, D), total # of cells (B, E) and CD4+ T cell # (C, F) in draining lymph nodes of mice immunized with OVA-CFA (A, B, C) or hALAC-CFA (D, E, F) in either the left hind footpad (open symbols) or left hind hock (closed symbols). Mice were injected in either the left hind footpad or hock with 10 μ g of the indicated proteins emulsified in CFA. Nine or ten days later, single cell suspensions of the draining lymph nodes (popliteal, sub-iliac and medial iliac) were counted, and CD4+ T cells were purified by negative selection. CD4+ T cell percentages in draining lymph nodes of footpad- and hock-immunized mice were compared by FACS analysis. Total cell counts in conjunction with CD4+ T cell percentages derived from FACS enabled calculation of CD4+ T cell number for each sample. * p<0.05, ** p<0.005, *** p<0.0005. Each symbol represents data from one mouse.

CD4+ T cell proliferation response

Fig. 3.

Lymph node CD4+ T cell proliferation response to OVA (A) and hALAC (B) Purified CD4+ T cells (50,000 per well) from draining lymph nodes of footpad (open symbols) or hock (closed symbols) immunized mice were cultured for four days in vitro with syngeneic irradiated T-depleted spleen cells (100,000 per well) purified from strain-matched control mice, and graded doses of the indicated protein antigens. Cultures were pulsed with tritiated thymidine during the last 12 hours of culture. Response to PPD, an estimate of the anti-mycobacterial response induced by CFA immunization, was also simultaneously measured in parallel cultures (insets) as a positive control for the immunization.

CD4+ T cell cytokine secretion response

Fig. 4.

Lymph node CD4+ T cell cytokine response to OVA(A) and hALAC(B). Purified CD4+ T cells (50,000 per well) from draining lymph nodes of footpad (open symbols) or hock (closed symbols) immunized mice were cultured in vitro with syngeneic irradiated T-depleted spleen cells (100,000 per well) purified from control mice, and graded doses of the indicated protein antigens. Response to PPD, an estimate of the anti-mycobacterial response induced by CFA immunization, was also simultaneously measured in parallel cultures (insets) as a positive control for the immunization. Supernatants were harvested after 72 hours to measure IFN- γ and IL-17 secretion using sandwich ELISA. * $p < 0.05$.

Fig. 5. Comparison of systemic OVA-specific (A) and hALAC-specific (B) IgG1, IgG2a (IgG2c in the case of C57Bl/6), Ig G2b and IgG3 levels in mice immunized with OVA-CFA (A) or hALAC-CFA (B) in either the left hind footpad (open symbols) or left hind hock (closed symbols). Each symbol represents data from one mouse. * $p < 0.05$.

Fig. 6.

BALB/cAnNCr (n=9; 4 footpad; 5 hock) and B10.D2 (n=5 each for footpad and hock) mice were injected with OVA-CFA in either the left hind footpad (open symbols) or the left hind hock (closed symbols). The effect of the injection on the mobility of the mice was scored daily for 12 days by animal facility staff in a blinded fashion using the scoring system described in the Materials and Methods. *** $p < 0.0005$.